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Effects of Subinhibitory Concentrations of Menthol on Adaptation, Morphological, and Gene Expression Changes in Enterohemorrhagic *Escherichia coli*

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Menthol ($C_{10}H_{20}O$) possesses antibacterial activity; nevertheless, bacterial adaptation to this compound has never been studied. Here we report that precultivation of enterohemorrhagic *Escherichia coli* (EHEC) strains in increasing subinhibitory (SI) concentrations of menthol significantly elevates (4- to 16-fold) their resistance to menthol. Concomitant morphological alterations included the appearance of mucoid colonies and reduced biofilm production. Scanning electron microscopy (SEM) examination revealed suppressed curli formation in menthol-adapted cells. Expression of the gene *cpsB10* (encoding one of the enzymes responsible for colanic acid production) was elevated in response to SI concentrations of menthol in a laboratory *E. coli* strain, whereas expression in an *rcsC* null mutant was reduced, implicating a partial role for the Rcs phosphorelay system in mediating the menthol signal. Adaptation to menthol also reduced expression of the locus of enterocyte effacement-encoded regulator (Ler). This reduction, together with reduced curli and biofilm formation and elevated mucoidity, suggests a general reduction in bacterial virulence following adaptation to menthol. Our results thus suggest menthol as a potential lead in the recently emerging alternative strategy of targeting bacterial virulence factors to develop new types of anti-infective agents.

The essential oil (EO) menthol ($C_{10}H_{20}O$; molecular weight, 156.27) is a cyclic monoterpene alcohol which gives plants of the genus *Mentha* their distinctive smell and flavor (32). It is used in oral hygiene products, confectionary, pharmaceuticals, cosmetics, and pesticides and as a food-flavoring agent, among other uses. With regard to its medicinal uses, menthol is available in both prescription and over-the-counter medications for a host of conditions, including gastrointestinal disorders, the common cold, respiratory conditions, and musculoskeletal pain (32). Moreover, both peppermint oil and menthol are active against a variety of microorganisms, including Gram-positive and Gram-negative bacteria as well as fungi (32, 39, 40). These activities are also common to other EOs, such as carvacrol and thymol, and are exploited in diverse products, including dental root canal sealers, antiseptics, food preservatives, and feed supplements (44–46). The antimicrobial activity of EOs is attributed mostly to their ability to integrate and disrupt bacterial membrane structure and function, although the exact mechanism of action is not fully understood (5, 44–46). Screening an *Escherichia coli* transposon library for altered tolerance to thymol revealed several genes involved in the bacterial response, suggesting that EOs might act at several sites simultaneously (40).

The emergence of antibiotic-resistant bacteria has raised interest in finding new antimicrobial agents. The natural source of EOs and their categorization as generally recognized as safe (GRAS) make them good candidates for antimicrobial agents and food preservatives (44–46). However, the possibility of bacterial adaptation to these compounds has barely been addressed (46, 47).

In this work, we examined several strains of the food-borne pathogen enterohemorrhagic *E. coli* (EHEC). Infection with this agent typically results in bloody diarrhea with no or low-grade fever and no leukocytes in the stool (22). Symptoms may progress, culminating in potentially fatal complications, such as hemolytic uremic syndrome (13, 22) and thrombotic thrombocytopenic purpura, in both the elderly and the young (13). This organism causes around 73,000 illnesses annually in the United States (37). Recently, organic sprouts contaminated with EHEC were identified as the cause of a deadly epidemic in Germany (20).

Several virulence factors contribute to the pathogenicity of EHEC strains, with the production of Shiga toxins being at the core of the infectious process (17). EHEC is a member of the attaching/effacing (A/E) pathogens, a group of Gram-negative bacteria defined by their ability to form A/E lesions on host intestinal cells (28). In these pathogens, most of the genes necessary for forming A/E lesions are located in the locus for enterocyte effacement (LEE) pathogenicity island (25). This locus contains genes for structural components of the secretion apparatus, which belongs to the type III secretion system, the adhesin intimin and its translocated receptor Tir, the translocon proteins EspA, EspB, and EspD, and secreted effecter proteins and their chaperones (8).

In this report, we demonstrate that gradual, sequential exposure of EHEC cells or a laboratory strain of *E. coli* to subinhibitory (SI) doses of menthol allows them to tolerate lethal doses of the substance. This adaptation was accompanied by an increase in colony mucoidity and a reduction in curli production and biofilm formation. The increase in mucoidity was accompanied by an increase in capsular polysaccharide B10 (*cpsB10*) gene expression, which is partially dependent on the Rcs phosphorelay. Adaptation also suppressed the expression of Ler, an activator of the LEE promoter in EHEC cells, implying a potential decrease in pathogenicity.

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TABLE 1 Bacterial strains and plasmid used in this study and MICs for menthol

Strain or plasmid	Relevant genotype and characteristics	Reference or source	MIC of menthol (mM)
E. coli strains			
933-TUV	EHEC O157:H7 derived from EDL933 by deletion of both the stx_1 and stx_2 genes	J. Leong	75
IR250	EHEC O157 8004 clinical isolate	I. Rosenshine	75
IR249	EHEC O157 clinical isolate	I. Rosenshine	75
ROWE	EHEC O157:H7; Rowe no. E304810	15	37.5
#85	EHEC O157 clinical isolate	The *E. coli* Reference Laboratory collection (Israel Ministry of Health, Jerusalem, Israel)	75
43894	EHEC O157:H7	ATCC	150
700728	EHEC O157:H7	ATCC	150
EC100	TransforMax EC100 *E. coli* F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80d*lacZ*ΔM15 Δl *acX74 recA1 endA1 araD139* Δ(*ara leu*)7697 *galU galK* λ$^-$ *rpsL nupG*	Epicentre, Madison, WI	75
IR3608	933-TUV cells containing pGY3607	I. Rosenshine (unpublished data)	75
SG20781	MC4100 *lon*$^+$ *cpsB10::lacZ* Mu-*imm*λ	38 (a gift from K. D. Young)	300
MZ63	*cps-lacZ rcsC*::kan	50 (a gift from D. Court)	300
Plasmids			
pGY3607	Contains the EHEC *LEE1* promoter and a mutated *ler* (to prevent autorepression and enhance GFP expression) and a *gfp* gene		

MATERIALS AND METHODS

Microorganisms, plasmids, media, and culture conditions. The *E. coli* strains and plasmids used in the study are listed in Table 1. All strains were routinely grown in tryptic soy broth supplemented with 0.5% (wt/vol) yeast extract (TSYE) (Difco, Detroit, MI) at 37°C under vigorous agitation (200 rpm) in an Innova 4000 shaker (New Brunswick Scientific, New Brunswick, NJ). For solid medium, agar (Difco) was added to a final concentration of 2% (wt/vol) (TSYEA).

Determination of MICs. MICs were determined using the broth dilution method as recommended by CLSI with minor modifications. Bacterial cultures of control or adapted cells were grown overnight in TSYE. Cells were washed twice with phosphate-buffered saline (PBS) by centrifugation and diluted in PBS to 10^5 CFU/ml, and 20 μl was used to inoculate 200 μl of TSYE containing 2-fold serial dilutions of menthol (Sigma, St. Louis, MO). The MIC was determined as the lowest concentration at which no visible growth occurred.

Adaptation conditions. Bacterial cultures were adapted by diluting a 24-h culture (~10^9 CFU/ml) to 10^4 CFU/ml in tubes containing TSYE (1 ml) with 2-fold serial dilutions of menthol ranging from 0 to 600 mM. These cultures were incubated as described above for an additional 24 h. Subcultures were prepared from the tube containing the highest concentration of menthol that resulted in turbidity after 24 h. Strains were subcultured in tubes containing increasingly higher concentrations of menthol until cells could grow in the presence of 600 mM menthol. Control cells were grown in medium without menthol for a similar period of time. Both adapted and control cells were kept in 15% glycerol at −80°C. A batch was thawed before each experiment and grown overnight in TSYE.

β-Galactosidase assay. *E. coli* strain SG20781 or MZ63 cells were grown in TSYE to an optical density at 600 nm (OD_{600}) of 0.1. Equal volumes were centrifuged (4,000 × *g*, 5 min) and resuspended in TSYE with or without 150 mM menthol. Samples were drawn at the indicated times, and β-galactosidase was assayed according to Miller's procedure (27). Briefly, cells were resuspended in chilled Z buffer (0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.1 M KCl, 0.001 M $MgSO_4$, 0.05 M β-mercaptoethanol; pH 7) and the OD_{600} was measured. Cells were permeabilized with 100 μl of chloroform and 50 μl of 0.1% SDS, and the tubes were incubated at 28°C for 5 min. Then 0.2 ml of *o*-nitrophenyl-β-D-galactoside (ONPG; 4 mg/ml in 0.1 M phosphate buffer) was added (time of addition was recorded), and the tubes were incubated at 28°C. When a yellow color developed, 0.5 ml of 1 M Na_2CO_3 was added to stop the reaction (time of addition was recorded). A 1-ml aliquot of this mixture was centrifuged (20,800 × *g*, 1 min), and the OD_{420} and OD_{550} of the supernatant were measured for use in the following equation, with T being the time elapsed between the addition of ONPG and the addition of Na_2CO_3 (min) and V being the volume of culture used in the assay (ml): Miller units = 1,000 × {[OD_{420} − (1.75 × OD_{550})]/(T × V × OD_{600})}. Experiments were repeated three times, and standard errors of the means from all experiments are shown. Tukey's multiple-comparison test was performed (using JMP, version 7; SAS Institute Inc., Cary, NC) to determine the statistical significance of the differences. *P* values were calculated and deemed statistically significant at *P* values of ≤0.05.

GFP assay. (i) Testing adapted cells. Overnight cultures of parental strain IR3608 and its isogenic menthol-adapted strain were brought to the same OD_{600}. Equal volumes were centrifuged (14,000 × *g*, 1 min) and resuspended in sterile double-distilled water (DDW). The OD_{600} and green fluorescent protein (GFP) levels were analyzed with a Synergy H4 multimode microplate reader (BioTek, Winooski, VT), illuminated with a xenon lamp, and set at excitation and emission wavelengths of 485 and 535 nm, respectively. Promoter activity was expressed by dividing the fluorescence by the OD_{600}. Experiments were repeated three times, and standard errors of the means from all experiments are shown. The significance of the difference between the control and adapted cells was calculated by a *t* test (using GraphPad Prism, version 2[3]; http://www.graphpad.com/prism/Prism.htm) and deemed statistically significant at a *P* value of ≤0.05.

(ii) Testing cells during exposure to menthol. Strain IR3608 cells were grown to an OD_{600} of 1. Equal volumes were centrifuged (4,000 × *g*, 5 min) and resuspended in TSYE with or without 18.75 mM menthol. Samples were drawn at the indicated times, washed, and resuspended in M9 medium containing 0.4% (wt/vol) glucose and 0.2% (wt/vol) Casamino Acids, and the OD_{600} and GFP levels were analyzed as indicated above. Experiments were repeated three times, and standard errors of the means from all experiments are shown. The significance of the difference between the treated and nontreated cells at each time point was calculated by a *t* test (using GraphPad Prism, version 2[3]) and deemed statistically significant at a *P* value of ≤0.05.

Curli formation. *E. coli* cells were streaked on solid LB without salts (10 g/liter tryptone, 5 g/liter yeast extract, 1.5% Difco agar) that was supplemented with Congo red (40 μg/ml; Sigma) and Coomassie brilliant blue G (20 μg/ml; Sigma). Plates were incubated at either 37°C or 26°C for 48 h.

SEM. *E. coli* cells were plated on TSYEA. Fresh (24 to 36 h of incubation) single colonies were picked to serve as samples. Samples were fixed with 5% (vol/vol) glutaraldehyde in PBS for 2 h. Dehydration was in a graded ethanol series, followed by critical-point drying with carbon dioxide. Samples were sputter coated with gold prior to examination in a scanning electron microscope (JSM 5140LV) operating at 20 kV. Images were recorded with an Eagle 2k × 2k charge-coupled device (CCD) camera (FEI). To obtain representative micrographs, 20 different fields from each treatment were examined.

Biofilm formation assay. Detection and quantitation of biofilms on polystyrene were performed in 96-well polystyrene microtiter plates (Nunc A/S, Roskilde, Denmark) using the procedure of Danese et al. (7) with some modifications. Tested strains were grown overnight at 37°C in TSYE, diluted in LB without salt to an OD_{600} of 0.05, and then plated in six replicate wells. Plates were incubated for 72 h at either 37°C or 26°C, washed three times with distilled water (DW), left to dry for 15 min at 30°C, stained with 100 μl of 1% (wt/vol) crystal violet (Sigma) for 15 min at room temperature, and rewashed three times by sinking the plate in DW. The bound dye was solubilized in 100 μl of 33% (vol/vol) acetic acid, and OD_{600} values were determined using an ELx808 microplate reader (BioTek). Percent biofilm formation was calculated to compensate for differences in biofilm formation between the different stains by using the following formula, where (i) is control or menthol-adapted cells: % biofilm formation = average OD_{600} (i)/average OD_{600} (control) × 100. Experiments were repeated three times, and standard errors of the means from all experiments are shown.

RESULTS AND DISCUSSION

Adaptation. Adaptation of bacteria to EOs is hampered by the latter's nonspecific disruption of the cell membrane (5, 44) in addition to their activity as multitarget antibacterial components (40). However, sequential passages in the presence of SI concentrations of menthol yielded adapted variants. The MICs of menthol for control cells are shown in Table 1. After adaptation, all strains exhibited MICs of >600 mM (higher menthol concentrations could not be reached due to solubility capacity), indicating a 4- to 16-fold increase. Replicated experiments revealed a similar pattern. Transferring menthol-adapted EHEC cells to TSYE broth in the absence of menthol for 7 days returned their menthol tolerance to the level of wild-type cells, supporting the claim that this is indeed an adaptation process and not acquired resistance.

Of all plant compounds possessing antimicrobial activity, EOs have been the most studied. Although their mode of action is not yet fully understood, their nonspecific, multitarget activity has led to the assumption that adaptation of bacteria to EOs is unlikely (5). Indeed, the very few attempts made to adapt bacteria to EOs were only moderately successful (46, 47). The most widely used method for adaptation of microorganisms to antimicrobial substances is through gradual and sequential transfers in media with progressively higher SI doses of the inhibitory factor (31). In this study, continuous growth of EHEC cells, as well as *E. coli* laboratory strains, in increasing concentrations of menthol resulted in a 4- to 16-fold increase in menthol tolerance of the adapted cells. To the best of our knowledge, this is the first time that bacterial adaptation to a monoterpene has been reported. The fact that both EHEC strains and a laboratory *E. coli* strain underwent adaptation suggests that this phenomenon is also relevant to other *E. coli* strains.

Changes in bacterial morphology. Adapted EHEC 933-TUV and *E. coli* EC100 cells formed bigger and more mucoid colonies (Fig. 1A). Similar results were obtained for all tested EHEC strains except strain 43894. In light of the consistent differences in mucoidity of most menthol-adapted cells, we examined possible alterations in cell morphology which are often associated with modifications in cell surface components (12). Figure 1B shows scanning electron micrographs of representative cells of menthol-adapted and nonadapted 933-TUV and EC100. The SEM analysis revealed that while 933-TUV control cells express high numbers of fimbriae and EC100 cells express somewhat fewer fimbriae, menthol adaptation diminished fimbria formation in both strains. To examine whether these fimbriae were curli, a Congo red binding assay was performed (30). For most laboratory strains of *E. coli*, curli expression is highest at temperatures below 30°C. However, it has been shown that many clinical strains of *E. coli*, including sepsis isolates, can express curli at 37°C (3); we therefore examined curli expression at both 26°C and 37°C. As seen in Fig. 1C, at 37°C, control cells of both 933-TUV and EC100 formed red colonies, indicative of Congo red binding, while adapted cells formed white colonies which did not bind Congo red. Table 2 summarizes the results of the Congo red binding assay for the different strains at different temperatures. At 37°C, control cells of six out of the eight strains tested bound Congo red and six of the strains lost this ability after adaptation to menthol. Similar results were obtained at 26°C, at which control cells of five out of the eight strains tested bound Congo red and four of the strains lost this ability after adaptation to menthol. The results obtained from the SEM and Congo red assay prove that menthol-adapted cells produce fewer fimbriae than their control counterparts and that at least some of these are curli fimbriae.

A major way in which microorganisms cope with their environment is to alter their morphology (49), and various morphological adaptations have been reported and reviewed (49). In response to nutrient limitation, some bacteria, such as *Bacillus subtilis*, enter into a complex developmental pathway that leads to the formation of spores (11). In the stationary phase, *Caulobacter crescentus* cells undergo pronounced morphological changes (48). Adaptation of *Staphylococcus aureus* to epigallocatechin gallate leads to suppressed separation of daughter cells and a 2-fold increase in cell wall thickness (4). Curli fibers are the major proteinaceous components of the complex extracellular matrix produced by many *Enterobacteriaceae* (3). Curli fibers are involved in adhesion to surfaces, cell aggregation, and biofilm formation. They also mediate host-cell adhesion and invasion and are potent inducers of the host inflammatory response (3). The regulation of curli gene expression is extremely complex (14), and little attention has been given to the effect of adaptation to SI stress on curli production. An increase in curli formation has been reported in adaptation of *Salmonella enterica* serovar Typhimurium (10) and exposure to SI conditions of the *E. coli* K-12 strain (33). In contrast to those results, we found that adaptation to menthol reduces curli production.

Curli fibers play a major role in biofilm formation (3), and thus the ability of adapted cells to form biofilms was compared to that of control cells using the microtiter plate assay (7). At 26°C, adaptation of the cells to menthol was accompanied by a significant reduction in the biofilm production ability of five out of the eight strains tested. At this temperature, strain 700728 did not form biofilm at all, while strains 43894 and #85 did not show any significant difference from the controls. An even greater reduction was observed at 37°C (Fig. 2): strain #85 did not produce any

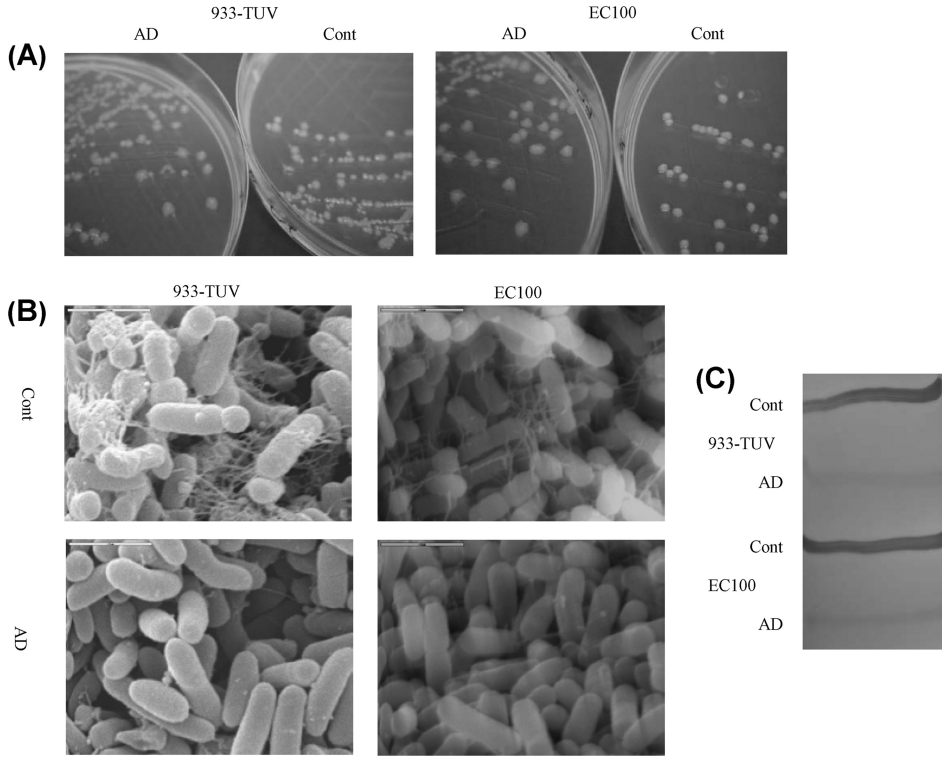

FIG 1 Morphological changes in *E. coli* cells after adaptation to menthol. (A) Colony morphology of control (Cont) and menthol-adapted (AD) *E. coli* cells grown on TSYEA. (B) Scanning electron micrographs of control (Cont) and menthol-adapted (AD) *E. coli* cells (×20,000 magnification). Scale bar = 2 μm. (C) Congo red binding assay. Control (Cont) and menthol-adapted (AD) *E. coli* cells were streaked on LB plates without salt that contained the curli-binding stains Congo red and Coomassie brilliant blue.

biofilm, while ROWE and 43894 showed no significant difference from the controls. Different groups have reported either enhancement (33) or reduction (21) of biofilm formation in the presence of SI concentrations of antimicrobial agents. Menthol may act via a mechanism similar to that of the agents found to cause reduced biofilm-forming ability. Overall, adaptation to menthol led to EHEC cells with increased mucoidity, diminished curli fiber production, and decreased biofilm formation.

Activity of the *cpsB10* gene. Colony mucoidity in *E. coli* arises via induced transcription of the *cps* gene cluster (38). We therefore tested whether exposure of an *E. coli* reporter strain (which was available in the laboratory) to SI levels of menthol would induce expression of the *cps* genes. *E. coli* strain SG20781 contains a

TABLE 2 Congo red binding of control and adapted cells

Strain	Ability of cells to bind Congo red when incubated at each temp (°C)			
	37		26	
	Control	Adapted	Control	Adapted
933-TUV	+	−	+	−
EC100	+	−	+	−
IR249	+	−	+	−
IR250	+	−	+	−
#85	+	−	−	−
43894	+	+	+	+
700728	−	−	+	+
ROWE	−	−	−	−

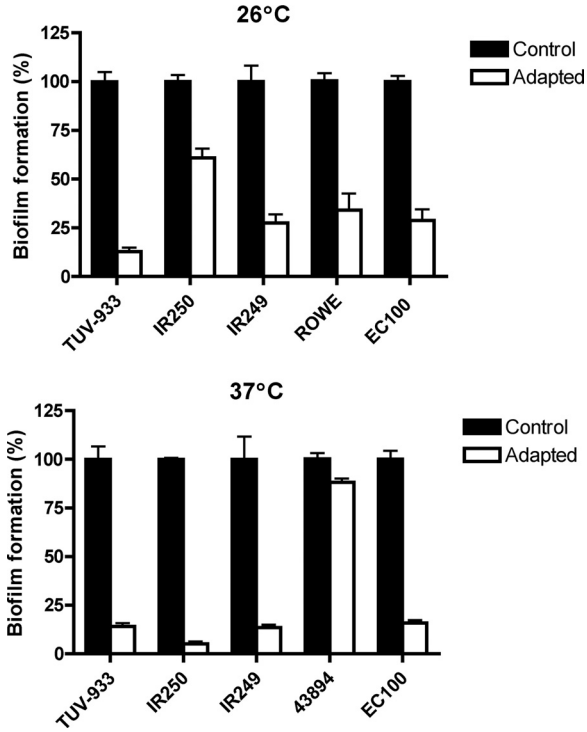

FIG 2 Biofilm formation by control and menthol-adapted strains. Control or menthol-adapted *E. coli* cells were grown for 72 h in a microtiter plate in medium enhancing biofilm formation at either 26°C or 37°C. Biofilm formation was quantified by crystal violet staining. Experiments were repeated three times, and standard errors of the means from all experiments are shown.

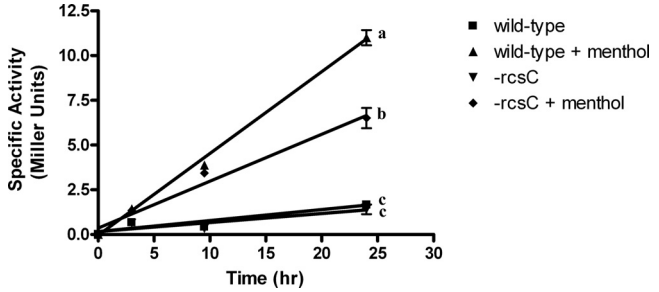

FIG 3 *cps-lacZ* induction by menthol. Menthol induction of a *cpsB*::*lacZ* fusion. ■, SG20781 (wild type) without menthol; ▲, SG20781 (wild type) with menthol; ▼, MZ63 (−*rcsC*) without menthol; ◆, MZ63 (−*rcsC*) with menthol. Specific activity is expressed in Miller units. Experiments were repeated three times, and standard errors of the means from all experiments are shown. Different letters indicate statistically significant differences in activity between strains at 24 h (by Tukey's multiple-comparison test; $P \leq 0.05$).

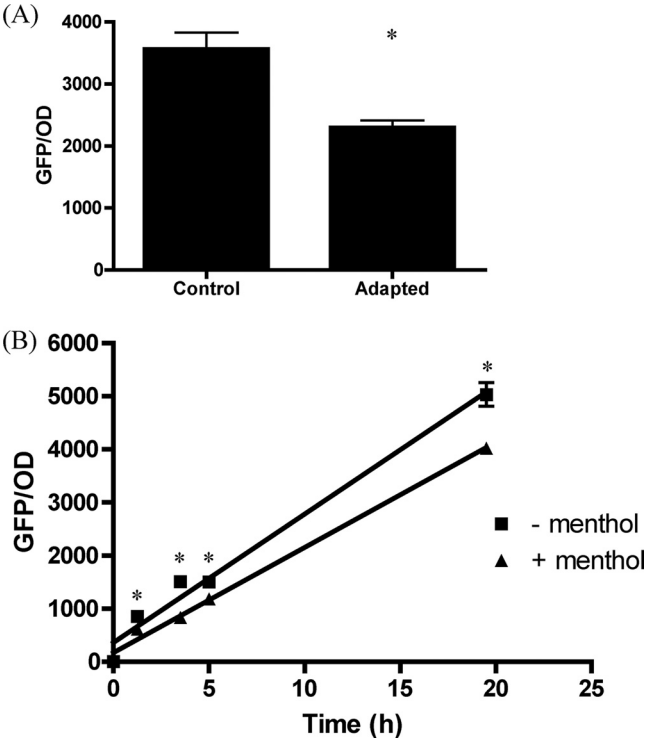

FIG 4 Activity of the *ler* promoter in response to SI menthol levels. Control and menthol-adapted TUV-933 cells containing pGY3607 (strain IR3608) were examined for their GFP activity using a BioTek Synergy H4 multimode microplate reader. Experiments were repeated three times, and standard errors of the means from all experiments are shown. (A) Effect of adaptation on P_{LEE1} activity. *, significant difference between control and adapted cells at a *P* value of ≤0.05 (*t* test). (B) Effect of menthol exposure on P_{LEE1} activity. *, significant difference between treated and nontreated cells at a *P* value of ≤0.05 (*t* test).

cpsB10::*lacZ* transcriptional fusion such that induction of the colanic acid structural genes can be monitored by measuring β-galactosidase levels. The MIC of menthol for SG20761 is presented in Table 1. SG20781 cells were grown in the presence of SI concentrations of menthol (one-half the MIC), and β-galactosidase levels were monitored over time. Growing SG20781 cells in the presence of SI menthol levels induced the *cpsB10* promoter after 3 h of exposure. This induction was enhanced over time (Fig. 3). Previous work has shown that exposing cells to desiccation (41), low temperature, high zinc concentrations, or biofilm formation (24) activates *cps* transcription. A similar result was obtained when *E. coli* cells were exposed to SI concentrations of β-lactam antibiotics (38). Exposing cells to SI levels of menthol might cause a similar stress and activate the *cps* genes via a similar mechanism.

Menthol activation of *cpsB10* is dependent on *rcsC*. The Rcs phosphorelay was first identified by its role in the transcriptional regulation of the *cps* genes in *E. coli*. It is a complex signal transduction system which is thought to be triggered by perturbations of the cell surface (among other signals) (24). Since one of the mechanisms of action suggested for menthol is perturbation of the cell surface (6), and following our results showing that menthol activates *cpsB10*, we checked whether this activation is mediated by the Rcs phosphorelay. We used strain MZ63 cells, which are SG20781 cells containing a null mutation in *rcsC*—the sensor kinase of the two-component system. We found that a null mutation in *rcsC* decreases the magnitude of *cpsB*::*lacZ* induction by menthol by about 1.8-fold after 24 h of exposure, a result which was found to be statistically significant by Tukey's multiple-comparison test ($P \leq 0.006$) (Fig. 3). These results indicate that *rcsC* and the Rcs phosphorelay are involved in mediating the signal transferred from menthol to the *cps* genes. Since a null mutation in *rcsC* did not abolish induction of the *cps* genes by menthol, we hypothesize that additional signal transduction systems are involved in the process. This is in accordance with previous work performed in our lab (40) which showed that menthol might act via several pathways simultaneously.

Activity of the *ler* promoter. Previous work has shown that the Rcs phosphorelay affects LEE gene expression in both positive and negative manners (43). LEE also encodes a regulator, Ler, which is necessary for the transcriptional activation of other LEE genes (26). Increased activity of the *ler* promoter (P_{LEE1}) has been reported after exposure to SI doses of antimicrobial compounds and environmental stresses (1). We hypothesized that adaptation to menthol might have a similar effect. To test this, we used EHEC cells containing a *ler*::*gfp* transcriptional fusion (IR3608). Unexpectedly, following adaptation to menthol, the adapted strain showed a 1.55-fold reduction in P_{LEE1} transcription (Fig. 4A). This result was found to be statistically significant by a *t* test ($P \leq 0.05$). To understand the timeline of this reduction in P_{LEE1} activity, we exposed wild-type IR3608 cells to SI levels of menthol (one-fourth the MIC) and monitored their OD_{600} and GFP levels at different time points. As seen in Fig. 4B, exposing the cells to SI levels of menthol for as little as 1 h reduced P_{LEE1} activity 1.4-fold, a statistically significant reduction ($P \leq 0.05$). This reduced level remained mostly constant during the time of exposure. LEE plays a major role in EHEC pathogenicity, and a reduction in P_{LEE1} activity *in vitro* might therefore also indicate a reduction of the adapted bacterium's pathogenicity *in vivo*. Moreover, the pronounced reduction in biofilm-forming ability and the increase in mucoidity of the adapted strains might also contribute to a reduction in virulence. Bacteria growing in biofilm show increased resistance to antimicrobials and are involved in chronic infection (18). Therefore, a reduction in biofilm-forming ability might reduce the risk from these bacteria and render them less pathogenic. The colanic acid capsule does not play a role in pathogenesis (24), and a high level of colanic acid expression seems to interfere with adhesion of uropathogenic *E. coli* (16). Thus, its overexpression by

menthol-adapted EHEC might also result in their reduced pathogenicity. In conclusion, we find that *in vitro*, the adaptation to menthol reduces P_{LEE1} activity and biofilm formation while increasing colanic acid expression. The implications of our *in vitro* observations for EHEC virulence reduction *in vivo* are uncertain. A determination of the amount of Shiga toxin and/or other major virulence factors produced by the adapted bacteria might serve to validate this hypothesis. Our results are in agreement with those of recent work showing that exposure of *S. aureus* (23, 34–36), *Shigella* strains (19), *Listeria monocytogenes* (42), *Klebsiella pneumoniae* (9), *E. coli*, *Vibrio harveyi* (29), and uropathogenic *E. coli* (16) to SI concentrations of EOs or their constituents reduces the expression of virulence factors. As interest rises in the targeting of bacterial virulence factors as an alternative strategy to develop new types of anti-infective agents, both our work and the reports cited above suggest that EOs, as well as the molecules forming them, might be good candidates to explore.

It has recently been reported that antibiotics not only are small molecules with therapeutic activity in killing or inhibiting microbial growth but can also act as signaling molecules affecting gene expression in bacterial communities (2). The results presented here, combined with those of previous studies, suggest that the same might be true for EOs and their constituents.

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